

(19)



Europäisches Patentamt

European Patent Office

Office européen des brevets



(11)

EP 0 471 758 B1

(12)

## EUROPEAN PATENT SPECIFICATION

XYIDS000132

(45) Date of publication and mention  
of the grant of the patent:  
11.09.1996 Bulletin 1996/37

(51) Int Cl. 6: G01N 33/483, A61K 35/52,  
G01N 21/64

(21) Application number: 90907724.0

(86) International application number:  
PCT/US90/02324

(22) Date of filing: 30.04.1990

(87) International publication number:  
WO 90/13303 (15.11.1990 Gazette 1990/26)

### (54) METHOD TO PRESELECT THE SEX OF OFFSPRING

VERFAHREN ZUR VORWAHL DES GESCHLECHTS DER NACHKOMMENSCHAFT

PROCEDE DE PRESELECTION DU SEXE DES PROGENITURES

(84) Designated Contracting States:  
AT BE CH DE DK ES FR GB IT LI LU NL SE

(30) Priority: 10.05.1989 US 349669

(43) Date of publication of application:  
26.02.1992 Bulletin 1992/09

(73) Proprietor: THE UNITED STATES OF AMERICA  
as represented by the Secretary UNITED STATES  
DEPARTMENT OF COMMERCE  
Washington, DC 20231 (US)

(72) Inventor: JOHNSON, Lawrence, A.  
Silver Spring, MD 20904 (US)

(74) Representative: Davies, Jonathan Mark  
Reddie & Grose  
16 Theobalds Road  
London WC1X 8PL (GB)

(56) References cited:  
WO-A-84/01265 GB-A- 2 145 112  
US-A- 4 083 957

- GAMETE RESEARCH, Volume 21, Number 4, issued 29 June 1988, JOHNSON et al., "Flow Sorting of X and Y Chromosome-Bearing Mammalian Sperm", pages 335-344, See the entire document.
- BIOLOGY OF REPRODUCTION, Vol.41, 12 July 1989, JOHNSON et al., "Sex Preselection in Rabbits: Live Births from X and Y Sperm Separated by DNA and Cell Sorting", See the entire document.
- Gamete Research, volume 15, No. 3, pages 267-283 (1986)
- Chemical Abstracts, volume 99, abstract number 2614w (1983)
- GAMETE RESEARCH, vol.16, no. 1, January 1987, NEW YORK, NY, US; L. A. JOHNSON ET AL.; pp. 1-9
- GAMETE RESEARCH, vol.17, no.3, June 1987, NEW YORK, NY, US; L.A. JOHNSON; pp. 203-212
- CHEMICAL ABSTRACTS, vol. 105, no.3, 21 July 1986, Columbus, Ohio, US; L.R. FRASER; abstract no. 22315V

Note: Within nine months from the publication of the mention of the grant of the European patent, any person may give notice to the European Patent Office of opposition to the European patent granted. Notice of opposition shall be filed in a written reasoned statement. It shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European Patent Convention).

**Description****Field of the Invention**

5 This invention relates to a method of preselecting the sex of offspring by sorting sperm into X and Y chromosome-bearing sperm based on differences in DNA content.

**Description of the Prior Art**

10 Gender of animal offspring is important to livestock producers. Because the dairy farmer has little use for most bull calves, the use of sexed semen to produce only females would make milk production more efficient. Swine farmers would produce pork more efficiently if they were able to market only female swine, because females grow faster than males.

15 In beef cattle and sheep breeds, the male grows at a faster rate than the female and hence is preferred for meat production.

20 In addition, the ability to specify male or female offspring should shorten the time required for genetic improvements, since desirable traits are often associated with one or the other parent. Planning the sex of cattle offspring is already practiced on a limited basis. This procedure consists of removing embryos from the cow, identifying their potential gender, and reimplanting only those of the desired gender. However, an ability to separate sperm into male-producing and female-producing groups before they are used for artificial insemination could enhance the overall value of offspring produced by embryo transfer.

25 Every living being has a set of paired chromosomes, which carry all the genetic material necessary to maintain life and also to propagate new life.

30 All but one pair of chromosomes are called autosomes and carry genes for all the characteristics of the body, such as skin, hair and eye color, mature size, and body characteristics. The remaining pair are called sex chromosomes. They carry the genetic material that specifies gender. One sex chromosome is called X, the other Y.

A sperm from the male or an egg from the female contains one of each pair of autosomes; in addition, in mammals the egg always contains an X chromosome, while the sperm always carries either an X or Y chromosome.

35 When a sperm and egg unite and the sperm carries the Y chromosome, the offspring is male (XY); however, if the sperm carries an X chromosome when it unites with the egg, the resulting offspring is female (XX).

The only established and measurable difference between X and Y sperm that is known and has been proved to be scientifically valid is their difference in deoxyribonucleic acid (DNA) content. The X chromosome is larger and contains slightly more DNA than does the Y chromosome. The difference in total DNA between X-bearing sperm and Y-bearing sperm is 3.4% in boar, 3.8% in bull, and 4.2% in ram sperm.

40 The amount of DNA in a sperm cell, as in most normal body cells, is stable. Therefore, the DNA content of individual sperm can be monitored and used to differentiate X- and Y-bearing sperm.

45 Since the difference in DNA mass in the sex chromosomes of most mammals is the only scientifically validated, measurable difference between X- and Y-bearing sperm, the chromosomal constitution [Moruzzi, J. Reprod. Fertile. 57: 319 (1979)] and/or measurement of DNA mass [Pinkel et al (1), Science 218: 904 (1982); Pinkel et al (2), Cytometry 3: 1 (1982); Johnson and Pinkel, Cytometry 7: 268 (1986); Johnson et al (1), Gam. Res. 16: 1 (1987); Johnson et al (2), Gam. Res. 17: 203 (1987)] are the only verifiable means other than fertility for determining sex-producing capability of a population of sperm. The literature describes many physical, biochemical, and functional methods that have purportedly sexed sperm [Amann and Seidel, "Prospects for Sexing Mammalian Sperm," Colorado Assoc. Univ. Press, Boulder (1982)]; several of these methods have been tested for relative DNA content [Pinkel et al., J. Anim. Sci. 60: 1303 (1985); Johnson (1), Theriogenology 29: 265 (1988)]. However, no method has been proven in controlled experiments to actually affect the sex ratio of offspring.

50 Previous studies have demonstrated that the difference in DNA content between X and Y chromosome-bearing sperm can be repeatedly measured and the sperm sex ratio of a sample of semen predicted [Johnson and Pinkel, supra; Johnson et al (1), supra; Johnson et al (2), supra; Johnson (1), supra; Johnson (2), Cytometry, Suppl. 2: 66 (Abstract) (1988)]. Verifiable separation by sorting of X and Y sperm based on DNA content has been accomplished with the vole [Pinkel et al (1), supra; Johnson, In "Beltsville Symposia in Agricultural Research X," P.C. Augustine, H. D. Danforth, & M.R. Bakst (eds.), Martinus Nijhoff, Boston, pp. 121-134 (1986)] and the chinchilla (Johnson et al (1), supra). However, preparation procedures damaged DNA viability. The sorting of sperm nuclei from several mammalian (bull, boar, ram, vole, chinchilla) species into separate X and Y chromosome-bearing populations at purities ranging from 92 to 99% has been accomplished [Johnson and Clarke, Gam. Res. 21: 335 (1988)]. Nuclear decondensation and pronuclear development was demonstrated in hamster eggs that had been microinjected with sorted X- or Y-bearing bull, boar, or ram sperm [Johnson and Clarke, supra].

55 WO 84/01265 discloses a method of sexing viable sperm cells using a cell cytometer in combination with selective

labelling of sperm cells with fluorescent dye. It suggests storing mammalian sperm at between 35° and 38.5°C prior to staining. Subsequently sperm are put in contact with a dye solution for a period of at least several minutes but usually for a period of at least 15 minutes to assure good dye penetration. Generally for mammalian sperm a cell membrane diffusion material such as DMSO or sperm-free human sperm is added so that dye can penetrate the sperm cells.

5 Chemical abstracts 105 (3), 22 315 V (1986; Fraser, L.R.) discloses that mouse sperm motility and fertilising ability in vitro could be improved by the introduction of low concentration (0.05 - 0.1 mg/ml) bovine serum albumin.

### SUMMARY OF THE INVENTION

10 It is an object of this invention to provide a method for sorting mammalian sperm into X and Y chromosome fractions based on DNA content.

It is a further object of this invention to teach a method of staining the DNA of mammalian sperm while maintaining viability of the sperm.

### DETAILED DESCRIPTION OF THE INVENTION

I have now demonstrated the separation, by flow sorting, of intact, viable X and Y chromosome-bearing rabbit and swine sperm populations based on relative DNA content; surgical insemination of the sorted sperm into does; and the subsequent birth of sexed offspring with a phenotypic sex ratio consistent with predictions based on the relative DNA content of the sorted sperm populations.

20 A flow cytometer measures the amount of fluorescent light given off when the sperm, previously treated with a fluorescent dye, pass through a laser beam. The dye binds to the DNA. The fluorescent light is collected by an optical lens assembly; the signal is transported to a photomultiplier tube, amplified and analyzed by computer. Because the X chromosome contains more DNA than the Y chromosome, the female sperm (X) takes up more dye and gives off more fluorescent light than the male sperm (Y).

25 For small differences in DNA to be detected between X and Y, the sperm must pass single file through the laser beam, which measures the DNA content of individual sperm.

In orthogonal flow cytometry, a suspension of single cells stained with a fluorochrome is made to flow in a narrow stream intersecting an excitation source (laser beam). As single cells pass through the beam, optical detectors collect 30 the emitted light, convert the light to electrical signals, and the electrical signals are analyzed by a multichannel analyzer. The data are displayed as multi-or single-parameter histograms, using number of cells and fluorescence per cell as the coordinates.

35 In order to use an orthogonal flow cytometric system to differentiate between X- and Y-bearing sperm DNA, a beveled sample injection tip and a second fluorescence detector in the forward position is required [Johnson and Pinkel, supra]. This paper is herein incorporated by reference. The modified system allows one to control the orientation of the flat ovoid sperm head as it passes the laser beam. Elimination of the unoriented sperm by electronic gating enhances precision. Typically, 80% of sperm nuclei (without tails) are properly oriented as they pass the laser beam.

40 In the modified Epics V (Registered Trade Mark) flow cytometer/cell sorter, hydrodynamic forces exerted on the flat, ovoid mammalian sperm nuclei orient the nuclei in the plane of the sample stream as they exit the beveled injection tip. Fluorescent signals are collected simultaneously by 90 and 0 degree optical detectors, from the edge and flat side of the sperm nucleus, respectively. For sorting, the sample stream is broken into uniform droplets by an ultrasonic transducer. Droplets containing single sperm of the appropriate fluorescence intensity are given a charge and electrostatically deflected into collection vessels. The collected sperm nuclei then can be used for microinjection into eggs. Since 45 the sperm nuclei have no tails, they cannot be used for normal insemination.

45 Accurate measurement of mammalian sperm DNA content using flow cytometry and cell sorting is difficult because the sperm nucleus is highly condensed and flat in shape, which makes stoichiometric staining difficult and causes stained nuclei to have a high index of refraction. These factors contribute to emission of fluorescence preferentially from the edge or thin plane of the sperm nucleus. In most flow cytometers and sorters, the direction of sample flow is orthogonal to the direction of propagation of the laser beam and the optical axes of the fluorescence detection. Consequently, fluorescence measurement is most accurate when the sperm fluorescence is excited and measured on an axis perpendicular to the plane of the sperm head [Pinkel et al (2), supra]. At relatively low sample flow rates, hydrodynamics are used to orient tailless sperm so that DNA content can be measured precisely on 60 to 80% of the sperm passing in front of the laser beam. The modified Epics V (RTM) system used in this study can measure the DNA content of tailless sperm from most species at the rate of 50 to 150 sperm per second [Johnson and Pinkel, supra].

55 Intact sperm (with tails), whether viable or nonviable, cannot be oriented as effectively as tailless sperm nuclei [Johnson (2), supra]. However, a 90-degree detector can be used to select the population of properly oriented intact sperm to be measured by the 0 degree detector. Since no hydrodynamic orientation is attempted, the sample flow rate can be much higher, which compensates somewhat for the fact that only 15 to 20% of intact sperm pass through the

laser beam with proper orientation. In this invention, the overall flow rate was approximately 2500 intact sperm per second. The intact X-and Y-bearing sperm fractions were sorted simultaneously from the population of input sperm at a rate of 80-90 sperm of each type per second.

It is, of course, of critical importance to maintain high viability of the intact sperm during the sorting process and during storage after sorting but prior to insemination.

Of the factors involved in maintaining sperm viability, the method of staining the sheath fluid, and the collecting fluid have been found to be especially important.

A nontoxic DNA stain must be selected. A preferred stain is Hoescht bisbenzimide H 33342 fluorochrome (Calbiochem-Behring Co., La Jolla, CA). To our knowledge, this fluorochrome is the only DNA binding dye that is nontoxic to sperm. Concentration of the fluorochrome must be minimal to avoid toxicity, and yet be sufficient to stain sperm uniformly and to detect the small differences in the DNA of X and Y sperm with minimal variation. A suitable concentration was found to be 5 µg/ml, but this may be varied from 4 to 5 µg/ml.

The sperm must be incubated with stain at sufficient temperature and time for staining to take place, but under mild enough conditions to preserve viability. Incubation for 1 hour at 35°C would also be effective. Incubation time has to be adjusted according to temperature; that is, 1.5 hr. for 30°C; 1 hr for 39°C.

Sheath fluid used in sorting cells must be electrically conductive and isotonic. A concentration of 10 mM phosphate buffered saline provided the necessary electrical properties, and 0.1% bovine serum albumin was added to enhance sperm viability by providing protein support for metabolism and viscosity for the sperm. The sheath fluid must be free of sugars and excess salts.

Dilution of sperm as occurs in sorting tends to reduce viability of the cells. To overcome this problem sperm were collected in test egg yolk extender [Graham et al, J. Dairy Sci. 55: 372 (1972)] modified by adjusting the pH and adding a surfactant. Details of the composition of the extender are shown in Example 1. The surfactant is believed to enhance capacitation of the sperm prior to fertilization.

To confirm the DNA content and predict the sex of the offspring of surgically inseminated X or Y sorted sperm fractions, an aliquot of the sorted sperm was sonicated to remove the tails, stained, and the nuclei was reanalyzed for DNA content to predict the proportion of X and Y sperm.

Although the detailed description which follows uses the sorting of rabbit sperm as an example of this invention, it is expected that the sperm of most mammals could be effectively sorted by following these procedures.

Rabbit semen was collected, diluted, and stained with a fluorochrome dye. Sperm were sorted in a modified Epics V (RTM) flow cytometer/cell sorter.

After being sorted, sperm were surgically inseminated into the uteri of rabbits.

The results obtained by surgical insemination of does with sorted intact sperm are presented in Table I. Recovery of ova 40 hr post-insemination indicated that stained sorted sperm, as well as unstained unsorted sperm, were capable of fertilizing rabbit ova in vitro.

Table I.

Fertilizing Capacity of Flow-Sorted Rabbit Spermatozoa After Intrauterine Insemination of Does				
Number of				
Treatment of Sperm	Does Inseminated	Ovulation Points	Eggs Recovered	Eggs Fertilized
Unsorted	2	16	9	9
Sorted	6*	59	46	39

\* One doe accounted for 7 recovered and 7 unfertilized eggs.

5  
10  
15  
20  
25  
30  
35  
40  
45  
50  
55

**Table II. Predicted and Actual Sex Ratios of Offspring After Intrauterine Insemination of Sorted X and Y Chromosome-bearing Rabbit Sperm**

Treatment of Sperm	Percentage and Numbers of Offspring					
	Number of Does		Predicted		Actual	
	Inseminated	Kindling	Total No. of Young Born	% Males	% Females	% Males (N) Females (N)
Sorted Y	16	5	21	81	19	81 (17) 19 (4)
Sorted X	14	3	16	14	86	6 (1) 94 (15)
Recombined X and Y	17	5	14	50	50	43 (6) 57 (8)
<b>Total</b>	<b>47</b>	<b>13</b>	<b>51</b>	—	—	<b>47 (24) 53 (27)</b>

Inseminations were also made to determine the comparability of predicted sex of offspring to phenotypic sex. As the data in Table II indicate, the predictability of the phenotypic sex based on DNA analysis of the separated intact sperm was very high. Reanalysis of the sorted Y population used for insemination indicated that 81% of the sperm

were Y-bearing. The sex ratio of offspring from these inseminations was identical to that predicted. These values were significantly different from theoretical 50:50 sex rates ( $P < .003$ ). Reanalysis of the sorted X-bearing sperm population used for insemination indicated that 86% were X-bearing and 14% were Y-bearing sperm. The phenotypic sex of the offspring from these inseminations was 94% female, which was different from the theoretical 50:50 ( $P < .0003$ ).

Inseminations were made with sorted X and Y populations that were recombined (recombined X and Y group) immediately before insemination. The assumption was made that the proportions of X and Y in the recombined samples were equal (50:50). The phenotypic sex resulting from the inseminations was 57% female and 43% male (Table II) and was not significantly different from the theoretical (50:50) sex ratio ( $P = .40$ ). The phenotypic sex ratio of offspring born of does inseminated with either sorted X-bearing or sorted Y-bearing sperm was different ( $P < .0002$  for X and  $P < .001$  for Y) from the theoretical (50:50) sex ratio expected from untreated semen.

Embryonic mortality was significant in the does inseminated with sorted intact sperm. With a reasonably high fertilization rate (Table I), one would expect a kindling rate of near 80% and litter size of about six from does of this age and breed. However, the kindling rate across the three treatment groups averaged 28%, with an average litter size of 3.9. The cause of the apparent high rate of embryonic death is thought to be due to the fluorochrome binding to the DNA and/or to the effect of the laser beam exciting the DNA bound fluorochrome. Earlier work has shown that sorted vole sperm nuclei that were microinjected into hamster eggs exhibited chromosome breakage in the developing sperm pronucleus [Libbus et al, Mut. Res. 182: 265 (1987)]. Those sperm had been sonicated, stained, sorted, and microinjected, a somewhat more rigorous treatment than the staining and sorting used in this study.

I have demonstrated that DNA can be used as a differentiating marker between X- and Y-bearing sperm, that DNA can be used to accurately predict the sex of offspring from separated X- and Y-bearing sperm populations, and that flow sorting is an effective means for separating viable X- and Y-bearing sperm populations suitable for production of offspring.

The following examples are intended only to further illustrate the invention and are not intended to limit the scope of the invention, which is defined by the claims.

#### Example 1

Semen was collected from mixed breed mature bucks by use of an artificial vagina. Sperm concentration was determined with a hemocytometer. The semen was diluted with Tris buffer, pH 6.9, to a concentration of  $10 \times 10^6$  per ml. Bisbenzimide H 33342 fluorochrome was added at a concentration of 5  $\mu\text{g}/\text{ml}$ . The samples were incubated for 1 hr at 35°C. Intact sperm were sorted on a modified EPICS V (RTM) flow cytometer/cell sorter. The stained intact sperm were excited in the ultraviolet (UV; 361 and 364 nm) lines of a 5-watt 90-5 Innova (RTM) Argon-ion laser operating at 2000mW. Data were collected as 256-channel histograms. Sheath fluid was 10 mM phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). Sperm were sorted into a test egg yolk extender.

The composition of the extender was N-tris(hydroxymethyl)-methyl-2-amino ethane sulfonic acid, 2.16 g; tris hydroxymethyl aminomethane, 0.51 g; dextrose, 0.1 g; streptomycin sulfate, 0.13 g; penicillin G, 0.08 g; egg yolk, 12.5 ml; Equex STM (Nova Chemical Sales, Scituate, MA), 0.5%; and distilled water, 50 ml. This mixture was centrifuged, and only the supernatant was used. The sorted sperm were concentrated by incubating at room temperature for 1 hr, after which the more dilute fraction was removed and the remainder was used for insemination 1 to 4 hr later.

#### Example 2

Mature New Zealand White does were injected with 150 international units of human chorionic gonadotropin (HCG) to induce ovulation, which was expected to occur 10 hr later. Seven hours after treatment with HCG, the does were surgically prepared by injection with Ketamine hydrochloride containing acepromazine and anesthetized under halothane and oxygen. The uterus was exposed by midline incision, and 100  $\mu\text{l}$  of sorted or unsorted sperm was placed into the lumen of the anterior tip of each uterine horn through a 21-gauge needle. Standard management practices were used in caring for the rabbits. These does were sacrificed 40 hr post-insemination; uteri were flushed and recovered eggs evaluated. All fertilized eggs recovered were classified as morula. The results of these experiments are shown in Table I.

#### Example 3

Table II shows the results of inseminations made into the tip of the uterine horn: the number of does that kindled and the phenotypic sex of the offspring compared to the predicted sex. Predicted sex of offspring was based on reanalysis of sorted intact sperm to determine relative DNA content. For reanalysis, the sorted sperm was sonicated for 10 sec and centrifuged at 15,000 g, the supernatant was discarded, and the pellet was resuspended in 9  $\mu\text{M}$  bisbenzimide H 33342. Phenotypic sex of the offspring was determined soon after birth and confirmed at later ages up to 10

weeks. Recombined X and Y is the sorted X and Y sperm populations recombined immediately before insemination.

#### Example 4

5 Using the methods of Examples 1, 2, and 3, viable swine sperm was sorted into viable X and Y chromosome-bearing populations. Two litters (18 pigs) from surgically inseminated boar semen produced 88% females from X-sorted sperm and 67% males from Y-sorted sperm.

10 **Claims**

1. A method to preselect the sex of mammalian offspring comprising:
  - a) staining viable intact sperm collected from a male mammal with a fluorescent non-toxic dye capable of selectively staining DNA in living cells by incubating the sperm with the dye at a temperature in the range of 30°-39°C for a period of time sufficiently long for staining to take place uniformly but sufficiently short to preserve viability of the sperm;
  - b) passing the sperm into an electrically conductive and isotonic viability-supporting sheath fluid to form a suspension of sperm which are caused to flow singly in a stream of sheath fluid;
  - c) passing the sheath fluid containing the sperm before an excitation light source causing the stained DNA to fluoresce;
  - d) passing the sheath fluid containing the sperm through both a means for detecting the fluorescence of the stained DNA and also a cell sorting means, the means for detecting fluorescence having at least two detectors arranged such that a first detector determines orientation of sperm on the basis of magnitude of fluorescence and controls a second detector to measure the DNA content of sperm on the basis of magnitude of fluorescence of those sperm that have been determined to be in a preselected orientation;
  - e) selecting by said cell sorting means the sperm having a DNA content corresponding to a desired chromosome which will produce the desired gender of offspring, and separating the selected sperm from non-selected sperm; and
  - f) collecting the selected sperm in a viability-supporting collecting fluid.
2. The method of claim 1, wherein said mammal is a rabbit.
3. The method of claim 1, wherein said mammal is a swine.
4. The method of claim 1, wherein said mammal is a bovine.
5. The method of claim 1, wherein said incubation is at a temperature of about 39°C for a period of about 1 hour.
- 40 6. The method of claim 1, wherein said incubation is at a temperature of about 35°C for a period of about 1 hour.
7. The method of claim 1, wherein said incubation is at a temperature of about 30°C for about 1.5 hour.
- 45 8. The method of claim 1, wherein said sheath fluid is phosphate-buffered saline solution, the solution also containing 0.1% bovine serum albumin to enhance sperm viability.
9. The method of claim 1, wherein said collecting fluid is modified test egg yolk extender.
- 50 10. The method of claim 1, wherein said sperm are hydrodynamically oriented in the flow of sheath fluid prior to being passed before said light source.
11. The method of claim 1, wherein said sperm are hydrodynamically oriented in the flow of sheath fluid by passing the fluid in a narrow stream through and out of a bevelled injection tip prior to being passed before said light source.
- 55 12. A method of pre-determination of the sex of non-human mammalian offspring comprising:
  - a) preselecting sperm according to the method of claim 1; and
  - b) fertilizing an egg from a non-human female mammal of the same species as the non-human male mammal

with the selected sperm in the collecting fluid.

13. A method of pre-determination of the sex of mammalian offspring comprising;
  - 5 a) preselecting sperm according to the method of claim 1; and
  - b) fertilizing in vitro an egg from a female mammal of the same species as the male mammal with the selected sperm in the collecting fluid.
14. The method of claim 1, further comprising eliminating sperm which are not properly oriented with an electronic gating system before sorting by said cell sorting means.
15. The method of claim 1, wherein the flow of sperm through the cell sorting means is regulated by an ultrasonic transducer.
16. The method of claim 1, wherein said sperm are sorted on the basis of X- or Y-chromosome DNA content with about 90% efficiency.
17. The method of claim 1, wherein said sperm are hydrodynamically oriented in the flow of sheath fluid and sperm which are not properly oriented are eliminated by an electronic gating system prior to being passed before said light source.
18. The method of claim 1, wherein said dye is bisbenzimid H33342 fluorochrome.

## 25 Patentansprüche

1. Verfahren zum Vorwählen des Geschlechts von Säuger-Abkömmlingen, das umfaßt:
  - a) Färben von einem männlichem Säuger erhaltenem lebensfähigem intaktem Sperma mit einem fluoreszierenden nichttoxischen Farbstoff, der in der Lage ist, DNA in lebenden Zellen selektiv zu färben, indem das Sperma bei einer Temperatur im Bereich von 30 bis 39°C während einer Zeitspanne inkubiert wird, die ausreichend lang ist, damit ein gleichförmiges Färben stattfinden kann, die aber auch ausreichend kurz ist, damit die Lebensfähigkeit des Spermias erhalten bleibt,
  - b) Einführen des Spermias in eine elektrisch leitende und isotonische, die Lebensfähigkeit unterstützende Trägerflüssigkeit, um eine Suspension von Spermien herzustellen, die einzeln in der Trägerflüssigkeit transportiert werden,
  - c) Vorbeiführen der die Spermien enthaltenden Trägerflüssigkeit an einer Anregungslichtquelle, die das Fluoreszieren der gefärbten DNA bewirkt,
  - d) Hindurchführen der die Spermien enthaltenden Trägerflüssigkeit sowohl durch eine Einrichtung zum Nachweis der Fluoreszenz der gefärbten DNA und außerdem durch eine Zellensortiereinrichtung, wobei die Einrichtung zum Nachweis der Fluoreszenz mindestens zwei so angeordnete Detektoren aufweist, daß ein erster Detektor die Orientierung von Spermien auf der Basis der Fluoreszenzstärke bestimmt und einen zweiten Detektor zur Messung des DNA-Gehalts von Spermien auf der Basis der Fluoreszenzstärke dieser Spermien steuert, die als in einer vorgewählten Orientierung befindlich bestimmt worden sind,
  - e) mittels der Zellensortiereinrichtung Auswählen der Spermien, die einen einem gewünschtem Chromosom, das das gewünschte Nachkommengeschlecht erzeugt, entsprechenden DNA-Gehalt hat, und Abtrennen der ausgewählten Spermien von nichtausgewählten Spermien und,
  - f) Sammeln der ausgewählten Spermien in einer die Lebensfähigkeit unterstützenden Sammelflüssigkeit.
2. Verfahren nach Anspruch 1, wobei der Säuger ein Kaninchen ist.
3. Verfahren nach Anspruch 1, wobei der Säuger ein Schwein ist.

4. Verfahren nach Anspruch 1, wobei der Säuger ein Rind ist.

5. Verfahren nach Anspruch 1, wobei die Inkubation bei einer Temperatur von etwa 39°C während einer Zeitdauer von etwa einer Stunde erfolgt.

10 6. Verfahren nach Anspruch 1, wobei die Inkubation bei einer Temperatur von etwa 35°C während einer Periode von etwa einer Stunde erfolgt.

10 7. Verfahren nach Anspruch 1, wobei die Inkubation bei einer Temperatur von etwa 30°C während etwa 1,5 Stunden erfolgt.

15 8. Verfahren nach Anspruch 1, wobei die Trägerflüssigkeit phosphatgepufferte Salzlösung ist, und wobei die Lösung außerdem 0,1% Rinderserumalbumin zur Förderung der Spermienlebensfähigkeit enthält.

15 9. Verfahren nach Anspruch 1, wobei die Sammelflüssigkeit modifiziertes Testeigelb-Streckmittel ist.

20 10. Verfahren nach Anspruch 1, wobei die Spermien in der Trägerflüssigkeitsströmung hydrodynamisch orientiert werden, bevor diese an der Lichtquelle vorbeigeführt wird.

20 11. Verfahren nach Anspruch 1, wobei die Spermien in der Trägerflüssigkeitsströmung hydrodynamisch orientiert werden, indem die Flüssigkeit in Form einer engen Strömung durch eine kegelige Injektionsspitze hindurchgeführt wird, bevor sie an der Lichtquelle vorbeigeführt wird.

25 12. Verfahren zur Vorbestimmung des Geschlechts nichtmenschlicher Säugeternachkommen, das umfaßt:

25 a) Vorröhren von Spermien nach dem Verfahren nach Anspruch 1, und

30 b) Befruchten eines Eis aus einem nichtmenschlichem weiblichen Säugetier der gleichen Art wie das nichtmenschliche männliche Säugetier mit dem ausgewählten Sperma in der Sammelflüssigkeit.

30 13. Verfahren zur Vorbestimmung des Geschlechts von Säger-Nachkommen, das umfaßt:

35 a) Vorröhren von Spermien nach dem Verfahren nach Anspruch 1, und

35 b) In-Vitro-Befruchten eines Eis eines weiblichen Säugers der gleichen Art wie der männliche Säuger mit dem ausgewählten Sperma in der Sammelflüssigkeit.

40 14. Verfahren nach Anspruch 1, das außerdem mittels eines elektronischen Torsystems das Eliminieren von Spermien umfaßt, die nicht richtig orientiert sind, bevor das Sortieren in der Zellensortiereinrichtung stattfindet.

40 15. Verfahren nach Anspruch 1, wobei die Spermienströmung durch die Zellensortiereinrichtung durch einen Ultraschallwandler reguliert wird.

45 16. Verfahren nach Anspruch 1, wobei die Spermien auf der Basis des X- oder Y-Chromosomen-DNA-Gehalts mit etwa 90% Effizienz sortiert werden.

45 17. Verfahren nach Anspruch 1, wobei die Spermien in der Trägerflüssigkeit hydrodynamisch orientiert werden, und Spermien, die nicht richtig orientiert sind, mittels eines elektronischen Torsystems vor dem Vorbeiführen an der Lichtquelle eliminiert werden.

50 18. Verfahren nach Anspruch 1, wobei der Farbstoff Bisbenzimid-H33342-Fluorochrom ist.

#### Revendications

55 1. Procédé de présélection du sexe de la progéniture de mammifères comprenant:

a) la coloration de spermatozoïdes intacts viables recueillis chez un mammifère mâle avec une colorant fluo-

5           rescent non toxique capable de colorer, de manière sélective, l'ADN des cellules vivantes, en laissant incuber les spermatozoïdes et le colorant à une température comprise entre 30° et 39°C pendant une période de temps suffisamment longue pour que la coloration se mette en place uniformément, mais suffisamment courte pour préserver la viabilité des spermatozoïdes ;

10          b) le passage des spermatozoïdes dans un fluide gainant électriquement conducteur et isotonique, assurant la viabilité de ceux-ci, afin de former une suspension de spermatozoïdes que l'on fait s'écouler un par un dans un courant de fluide gainant ;

15          c) le passage du fluide gainant contenant les spermatozoïdes devant une source lumineuse d'excitation qui provoque la fluorescence de l'ADN coloré ;

20          d) le passage du fluide gainant contenant les spermatozoïdes à travers un moyen destiné à déceler la fluorescence de l'ADN coloré et également un moyen destiné à trier les cellules, le moyen destiné à détecter la fluorescence présentant au moins deux détecteurs disposés de façon qu'un premier détecteur détermine l'orientation des spermatozoïdes sur la base de l'amplitude de la fluorescence et commande un second détecteur destiné à mesurer la teneur en ADN dans les spermatozoïdes sur la base de l'amplitude de la fluorescence de ces spermatozoïdes qui ont été déterminés comme étant dans une orientation présélectionnée ;

25          e) la sélection dudit moyen destiné à trier les cellules, les spermatozoïdes ayant une teneur en ADN correspondant à un chromosome souhaité qui produira le sexe souhaité pour la progéniture, et la séparation desdits spermatozoïdes sélectionnés des spermatozoïdes non sélectionnés ; et

30          f) la récupération des spermatozoïdes sélectionnés dans un fluide de récupération assurant la viabilité.

2. Procédé selon la revendication 1, dans lequel ledit mammifère est un lapin.

3. Procédé selon la revendication 1, dans lequel ledit mammifère est un porc.

4. Procédé selon la revendication 1, dans lequel ledit mammifère est un bovin.

5. Procédé selon la revendication 1, dans lequel ladite incubation est effectuée à une température d'environ 39°C pendant une période d'environ 1 heure.

6. Procédé selon la revendication 1, dans lequel ladite incubation est effectuée à une température d'environ 35°C pendant une période d'environ 1 heure.

7. Procédé selon la revendication 1, dans lequel ladite incubation est effectuée à une température d'environ 30°C pendant une période d'environ 1,5 heure.

8. Procédé selon la revendication 1, dans lequel ledit fluide gainant est une solution saline tampon de phosphate, la solution contenant également de la sérum albumine de bovin à 0,1% pour augmenter la viabilité des spermatozoïdes.

9. Procédé selon la revendication 1, dans lequel ledit fluide de récupération est un agent diluant de jaune d'oeuf pour essai modifié.

10. Procédé selon la revendication 1, dans lequel lesdits spermatozoïdes sont orientés de manière hydrodynamique dans le flux du fluide gainant avant de passer devant ladite source lumineuse.

11. Procédé selon la revendication 1, dans lequel lesdits spermatozoïdes sont orientés de manière hydrodynamique dans le flux du fluide gainant en faisant passer le fluide dans un courant étroit à travers un embout d'injection biseauté avant de passer devant ladite source lumineuse.

12. Procédé de prédétermination du sexe de la progéniture de mammifères non humains, et qui comprend :

- la présélection des spermatozoïdes selon le procédé de la revendication 1 ; et
- la fécondation d'un oeuf prélevé sur un mammifère femelle non humain, et de la même espèce que le mammifère mâle non humain, avec les spermatozoïdes sélectionnés dans le fluide de récupération.

13. Procédé de prédétermination du sexe de la progéniture de mammifères qui comprend

- la présélection des spermatozoïdes selon le procédé de la revendication 1 ; et

b) la fécondation in vitro d'un oeuf prélevé sur un mammifère femelle de la même espèce que le mammifère mâle avec les spermatozoïdes sélectionnés dans le fluide de récupération.

5 14. Procédé selon la revendication 1, qui comprend en outre l'élimination des spermatozoïdes qui ne sont pas correctement orientés au moyen d'un système de portique électronique avant le tri par ledit moyen destiné à trier les cellules.

10 15. Procédé selon la revendication 1, dans lequel le flux de spermatozoïdes qui traverse le moyen destiné à trier les cellules est régulé par un capteur à ultrasons.

16. Procédé selon la revendication 1, dans lequel lesdits spermatozoïdes sont triés sur la base de la teneur en ADN du chromosome X ou du chromosome Y, avec une efficacité d'environ 90%.

15 17. Procédé selon la revendication 1, dans lequel lesdits spermatozoïdes sont orientés, de manière hydrodynamique dans le flux de fluide gainant et les spermatozoïdes qui ne sont pas correctement orientés sont éliminés par un système de portique électronique avant de passer devant ladite source lumineuse.

20 18. Procédé selon la revendication 1, dans lequel ledit colorant est un fluorochrome de bisbenzimide H 33342.

25

30

35

40

45

50

55